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Hydrogen peroxide biosensor based on a myoglobin/hydrophilic room temperature ionic liquid film

Afsaneh Safavi *, Fatemeh Farjami

Department of Chemistry, College of Sciences, Shiraz University, Shiraz 71454, Iran

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ABSTRACT

The composite film based on Nafion and hydrophilic room temperature ionic liquid (RTIL) 1-butyl-3-methyl-imidazolium chloride ([bmim]Cl) was used as an immobilization matrix to entrap myoglobin (Mb). The study of ionic liquid (IL)–Mb interaction by ultraviolet–visible (UV–vis) spectroscopy showed that Mb retains its native conformation in the presence of IL. The immobilized Mb displayed a pair of well-defined cyclic voltammetric peaks with a formal potential (E_o') of −0.35 V in a 0.1 M phosphate buffer solution (PBS) of pH 7.0. The immobilized Mb exhibited excellent electrocatalytic response to the reduction of hydrogen peroxide, based on which a mediator-free amperometric biosensor for hydrogen peroxide was designed. The linear range for the determination of hydrogen peroxide was from 1.0 to 180 μM with a detection limit of 0.14 μM at a signal/noise ratio of 3. The apparent Michaelis constant (K_m^{app}) for the electrocatalytic reaction was 22.6 μM. The stability, repeatability, and selectivity of the sensor were evaluated. The proposed biosensor has a lower detection limit than many other IL–heme protein-based biosensors and is free from common interference in hydrogen peroxide biosensors.

Hydrogen peroxide (H_2O_2)[1] determination is of great importance because of its essential role in food, industry, biology, pharmaceutical, and environmental research [1,2]. H_2O_2 is also the main product of most enzymatic reactions; thus, its detection is very interesting for the development of biosensors. Titrimetry [3], chemiluminescence [4], and spectrometry [5] are conventional methods for H_2O_2 detection. These methods are generally time-consuming, highly prone to interferences [6], and difficult for automation detection and can be unreliable for some food and biological samples due to the difficulty in obtaining a clear solution for final measurements. Electrochemical methods [7–10] can overcome the above drawbacks for the determination of H_2O_2. Amperometric biosensors are attractive because of their simplicity and high sensitivity. Many electrochemical techniques make use of the reduction of H_2O_2 by the catalysis of immobilized enzymes or protein to construct biosensors, which are based on direct electron transfer between an electrode and immobilized protein [11–14]. It is also well known that the study of direct electron transfer of redox proteins can establish a good model for understanding the complicated electron transfer mechanisms in biological systems and elucidating the relationship between their structures and biological functions. However, direct electron transfer between redox proteins and electrode is usually sluggish due to the deep burying of electroactive groups in the protein, denaturing caused by the serious adsorption of proteins at the electrode surface, and the unfavorable orientation of the protein immobilized at the electrode surface [15].

The iron heme protein, myoglobin (Mb), does not have major in vivo enzyme functions, but it has catalytic properties and can serve as a relatively low-cost "enzyme mimics." Various materials such as surfactants [16], polymers [17], and nanoparticles [18–20] have been exploited to modify the electrodes. For sensor applications, stable thin films retaining a high loading of native Mb on electrodes in an open network could lead to high sensitivity due to the fact that they facilitate direct electron transfer while not limiting mass transport.

Nafion, an ion exchange polymer that is very resistant to chemical attack (even by strong oxidants at elevated temperatures), has found increasing use as a membrane material. Because of its unique biocompatibility properties, Nafion has been used extensively for the modification of electrode surfaces used in biosensors and chemical sensors [21,22]. Ionic liquids (ILs) have many specific characteristics such as good chemical stability, high conductivity, and low volatility. By using ILs as a new matrix, protein electrochemistry is achieved in different IL-based composite film modified electrodes [23–25]. Doping room temperature ionic liquids (RTILs) in Nafion can improve its conductivity [26], whereas adding

* Corresponding author. Fax: +98 711 2286008.
E-mail address: safavi@chem.susc.ac.ir (A. Safavi).

[1] *Abbreviations used:* H_2O_2, hydrogen peroxide; Mb, myoglobin; IL, ionic liquid; RTIL, room temperature ionic liquid; HRP, horseradish peroxidase; GCE, glassy carbon electrode; [bmim]Cl, 1-butyl-3-methyl-imidazolium chloride; UV–Vis, ultraviolet–visible; CV, cyclic voltammogram; PBS, phosphate buffer solution; RSD, relative standard deviation.

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Nafion into RTILs can also improve its adhering ability to the electrode surface. Therefore, a combination of Nafion and ILs can provide a suitable composite film.

Chen and coworkers used Nafion-hydrophobic IL composite film as an immobilization matrix to entrap horseradish peroxidase (HRP) [26] and showed its electrocatalytic activity for the reduction of H_2O_2 and O_2.

It has also been reported that the water-miscible imidazolium-based ILs can interact with glassy carbon electrode and form molecular films on the electrode surface [27]. These molecular films are found to possess striking electrochemical properties such as electrocatalysis on oxidation of ascorbic acid and the capability to facilitate direct electron transfer in HRP, but it has very limited stability. In the current work, Nafion was used as a binder to adhere water-miscible IL [bmim] Cl on glassy carbon electrode (GCE) surface. It was found that the obtained Nafion–[bmim]Cl composite film provides a stable matrix to facilitate the direct electrochemistry of Mb and to catalyze the reduction of H_2O_2.

Materials and methods

Reagents

Nafion (perfluorinated ion exchange resin, 5 wt% solution in a mixture of lower aliphatic alcohols and water) was purchased from Aldrich and used as received. [bmim]Cl was purchased from Merck. Mb from horse heart, minimum 90% (Phast Gel), was purchased from Sigma and used as received. A 30% H_2O_2 solution was purchased from Riedel–de Haën, and a fresh stock solution of H_2O_2 (0.1 M) was prepared daily. All other reagents were of analytical reagent grade and used without further purification. All aqueous solutions were made with deionized water.

Electrode preparation

For preparation of Nafion/Mb/IL composite film, equal volumes of a 0.2 M solution of IL, [bmim]Cl, and 10 mg ml^{-1} Mb were mixed, and then 50 μl of this solution was mixed with an equal volume of Nafion and sonicated for 30 min until a homogeneous solution resulted. Then an aliquot of 3 μl of the mixture was coated on the electrode with a microsyringe and the electrode was stored for 10 h at 4 °C to acquire Nafion/Mb/IL composite film-modified GC electrode. The Nafion/IL/GCE was prepared using the same procedure as stated above without adding Mb. The Nafion/GCE was constructed without adding MB and IL. All of the prepared electrodes were washed with water before electrochemical measurements.

Apparatus

Voltammetric measurements were performed using an Autolab electrochemical system (Eco-Chemie, Utrecht, Netherlands) equipped with Autolab PGSTAT-302N and GPES software (Eco-Chemie). The electrochemical cell was assembled with a conventional three-electrode system. The working electrode used in this study was a GCE with 1 mm diameter. An Ag/AgCl/KCl (3 M) reference electrode (Metrohm) was used together with a platinum disk as a counter electrode. Before electrode preparation, the working electrode was polished using 0.05 μm aluminum oxide slurries and then washed carefully with distilled water. After polishing, the electrode was ultrasonicated in distilled water for approximately 5 min immediately before use. The IL–Mb interactions were followed by recording the spectra of the solution at different concentrations of IL using a Perkin Elmer Lambda 2 spectrophotometer.

Results and discussion

UV–vis spectroscopic characterization

Ultraviolet–visible (UV–vis) spectroscopy is an effective means to probe into the characteristic structure of proteins. Fig. 1 shows UV–vis spectra of Mb at increasing [bmim]Cl concentrations in phosphate buffer solution of pH 7.0. The spectrum of Mb, which has a sharp Soret band at 409 nm, Q bands at 504, and a CT1 band at 630 nm, is characteristic of a six-coordinated high-spin heme with a histidine residue (His93) and a water molecule bound at the fifth and sixth coordination positions of the iron atom, respectively [28]. In fact, these spectral features are considered as a spectral fingerprint of Mb and can provide very rich information on the heme environment and coordination. As can be seen from Fig. 1, with the addition of [bmim]Cl up to 0.22 M, no significant change in the spectrum of Mb was observed. It can be concluded that the presence of [bmim]Cl has no effect on the Mb conformation and does not cause any unfolding phenomenon in the Mb structure and that Mb has retained its bioactivity in the presence of [bmim]Cl. Due to the unique ionic-exchanging, film-forming, and biocompatible properties of Nafion [29–31], it is evident that Nafion/IL forms a suitable medium for the immobilization of Mb on GCE. Moreover, Nafion is a negatively charged film, and electrostatic interaction with positive charge of IL promotes the stability of the composite.

Direct electrochemistry of Mb at Nafion/Mb/IL/GCE

Fig. 2 shows cyclic voltammograms (CVs) of Nafion/Mb/IL/GCE (curve a), Nafion/IL/GCE (curve b), Nafion/GCE (curve c), and Nafion/Mb/GCE (curve d). A pair of well-defined, nearly symmetrical redox peaks were observed for Nafion/Mb/IL/GCE (curve a) corresponding to the electrochemical redox reaction between Mb–Fe(III) and Mb–Fe(II). The anodic (E_{pa}) and cathodic (E_{pc}) peak potentials were located at –0.332 and −0.364 V, respectively. The nearly symmetric oxidation and reduction peaks were separated by 0.32 V at a scan rate of 0.1 V s^{-1}. The formal potential as the midpoint between the reduction–oxidation peak pair of the heme Fe(III)/Fe(II) redox couple was −0.35 V. Under the same conditions and in the absence of Mb, Nafion/GCE or Nafion/IL/GCE did not show these characteristic redox peaks in the working potential range. The solution was deaerated by purging Ar gas, but because of residual impurity of oxygen, small peaks due to oxygen ap-

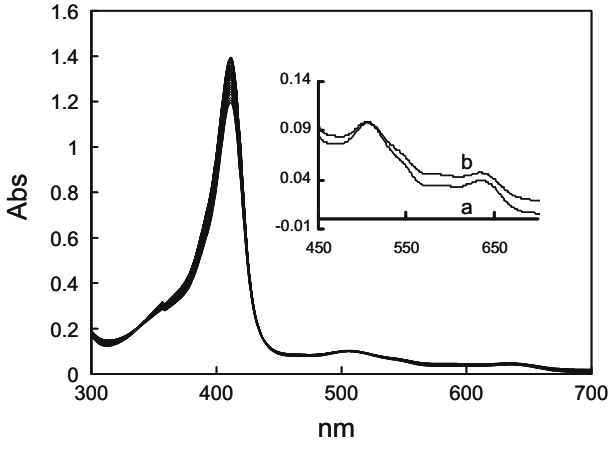

Fig. 1. UV–vis absorption spectra of Mb (1.24×10^{-5} M) in PBS (pH 7.0) by adding [bmim]Cl up to 0.22 M. The inset shows spectral changes in the Q-band region of Mb (1.24×10^{-5} M) in PBS (pH 7.0): (a) [bmim]Cl (0 M); (b) [bmim]Cl (0.22 M).

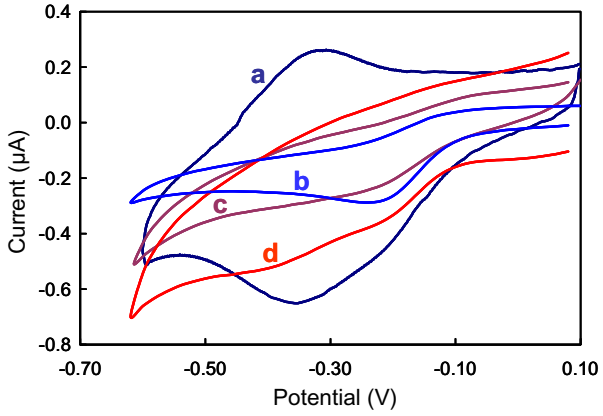

Fig. 2. CVs at different electrodes in 0.1 M PBS (pH 7.0) at a scan rate of 0.1 V s^{-1}: (a) Nafion/Mb/IL/GCE; (b) Nafion/IL/GCE; (c) Nafion/GCE; and (d) Nafion/Mb/GCE.

peared in Figs. 2b and 2c. The same reason caused the presence of a small shoulder in Figs. 2a and 2d.

Scan rate studies at Nafion/Mb/IL/GCE showed a slight peak shift in the scan rate range of 0.1 to 0.8 V s^{-1}. Such behavior is characteristic of nonideal, quasireversible thin-layer voltammetry of redox proteins [32]. The peak-to-peak separation value was much smaller compared to the corresponding values of other protein–RTIL film modified electrodes [23–25].

Fig. 2d shows the CV when Mb was immobilized on GCE by a Nafion film without the presence of IL. Although a small cathodic peak was observed, no anodic peak was seen at a scan rate of 0.1 V s^{-1}. As suggested by other researchers [26], this result shows that the presence of IL in the composite film can facilitate the direct electron transfer of Mb on the GCE.

Fig. 3A shows CVs for Nafion/Mb/IL/GCE studied at different scan rates from 0.1 to 0.8 V s^{-1}. The results show symmetrical anodic and cathodic peaks of approximately equal heights for Mb at different scan rates. The anodic and cathodic peak currents were linearly related to the scan rate (v) in the range of 0.1 to 0.8 V s^{-1} with regression coefficients of 0.9934 and 0.9946 for anodic and cathodic peak currents, respectively (Fig. 3B). This indicates that the electron transfer process for Nafion/Mb/IL/GCE is a surface-controlled one. The logarithm of the anodic and cathodic peak currents (log i_{pa} and log i_{pc}, respectively) versus logarithm of scan rate (log v) gave linear relationships with slopes of 1.08 and 1.07 for anodic and cathodic currents, respectively. These values are very close to the theoretical value of 1 expected for thin-layer electrochemical behavior [33,34]. That is, all of the electroactive sites of Mb, Fe(III), are converted to Fe(II) on the forward cathodic scan with full conversion of Fe(II) to Fe(III) on the reverse anodic scan [35–37].

Surface coverage (Γ^*) of the electroactive Mb adsorbed on the Nafion/Mb/IL/GCE modified electrode was measured by integrating CVs at low scan rates and was calculated as 5.89×10^{-11} mol cm^{-2}. This result is larger than the theoretical monolayer coverage of 1.58×10^{-11} mol cm^{-2} for Mb [38], showing that several layers of Mb are entrapped in the composite film participating in the electron transfer process.

In most cases, protein redox behavior is often significantly dependent on the solution pH. CVs of Nafion/Mb/IL/GCE were studied in solutions with varying pH values in the range of 2.0 to 9.0 (Fig. 4). An increase of solution pH leads to a negative shift in potential for both reduction and oxidation peaks for the modified electrode. The potential has a linear relationship with pH from 2.0 to 9.0 with a slope of −48.3 mV/pH (inset of Fig. 4). This suggests that a single protonation accompanies a single electron transfer between the electrode and the heme of Mb, represented in general terms by

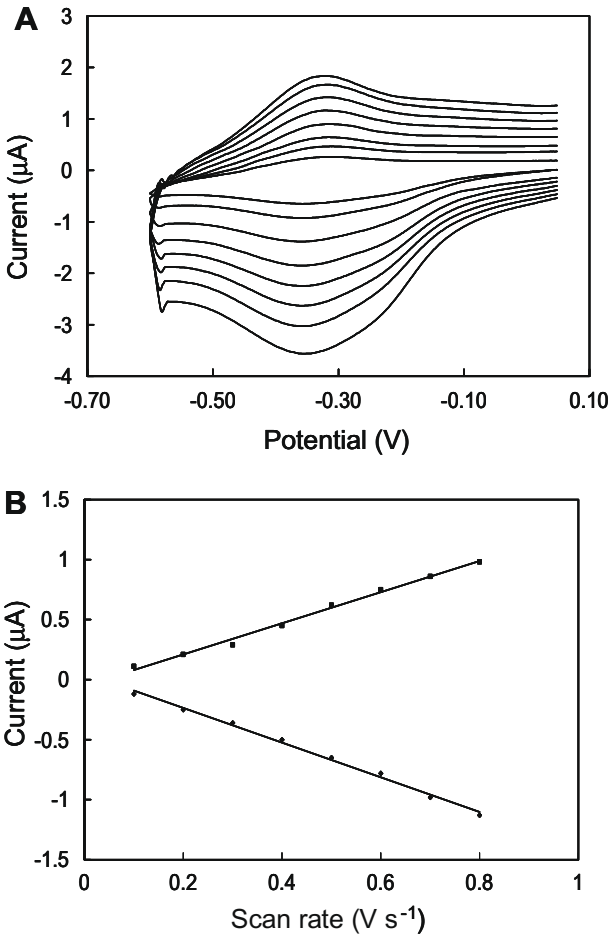

Fig. 3. (A) CVs of Nafion/Mb/IL/GCE in 0.1 M PBS (pH 7.0) at different scan rates (from inside to outside): 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 V s^{-1}, respectively. (B) Plot of cathodic and anodic peak currents versus scan rate.

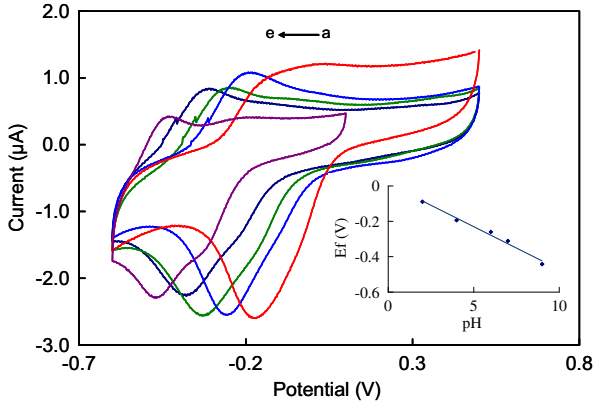

Fig. 4. CVs of Nafion/Mb/IL/GCE measured in different pH values at 0.5 V/s. The inset shows linear dependence of the formal potential versus pH values. (a) pH 2; (b) pH 4; (c) pH 6; (d) pH 7; (e) pH 9.

$$\text{Mb-heme Fe(III)} + H^+ + e^- \rightarrow \text{Mb-heme Fe(II)}.$$

Electrocatalytic reduction of H_2O_2 at Nafion/Mb/IL/GCE

The electrocatalytic effect of Nafion/Mb/IL/GCE modified electrode on the reduction of H_2O_2 was investigated. Fig. 5 shows the CVs of the Nafion/Mb/IL/GCE in a 0.1 M phosphate buffer solution

(PBS, pH 7.0) in the absence (curve a) and presence (curve d) of 0.1 mM H_2O_2 at a scan rate of 0.1 V s^{-1}. When H_2O_2 was added to PBS (pH 7.0), an increase in reduction peak was seen at approximately −0.22 V with the disappearance of the oxidation peak for the couple of Mb Fe(II)/Fe(III). However, no direct reduction peak was observed at the Nafion/IL/GCE in the absence (curve b) or presence of H_2O_2 in the same potential range (curve c). The mechanism of catalytic reduction of H_2O_2 can be expressed as

$$\text{Mb-heme Fe(III)} + H^+ + e^- \rightarrow \text{Mb-heme Fe(II)}$$

$$H_2O_2 + 2H^+ + 2\text{Mb-heme Fe(II)} \rightarrow 2\text{Mb-heme Fe(III)} + 2H_2O.$$

In the presence of H_2O_2, Mb–heme Fe(II) is efficiently converted to its oxidized form, Mb–heme Fe(III). Consequently, Mb–heme Fe(III) is reduced at the electrode surface by the direct electron transfer. The CVs obtained for a series of H_2O_2 solutions with various concentrations are illustrated in Fig. 6.

The Nafion/Mb/IL/GCE can also be used as an amperometric sensor for the determination of H_2O_2 in solution. With the successive addition of H_2O_2 to continuous stirring PBS (pH 7.0), the amperometric response of Nafion/Mb/IL/GCE to H_2O_2 was recorded under a potential of −0.45 V (Fig. 7). A stepwise growth of reduction current can be observed with increasing amounts of H_2O_2 in PBS. In addition, the time required to reach 95% of the steady-state current was less than 5 s after the addition of H_2O_2. This result shows that the response time of the sensor is short. The proposed biosensor showed a linear calibration range for H_2O_2 from 1.0 to 180 μM M [I (μA) = 0.0767 + 0.0146 C (M), R^2 = 0.9934, n = 10] (inset of Fig. 7). The detection limit (signal/noise = 3) for the determination of H_2O_2 was obtained as 0.14 μM. The higher performance of this electrode in comparison with other protein–RTIL film modified electrodes is obvious from Table 1. The film preparation is simple and convenient; its detection limit is lower than the detection limits of some electrodes modified with various materials such as nanomaterials [7,9,10].

When the concentration of H_2O_2 is higher than 180 μM, a response plateau in calibration curve is observed, showing the characteristics of the Michaelis–Menten kinetic mechanism [39]. The apparent Michaelis–Menten constant K_m^{app} can be obtained from the electrochemical version of the Lineweaver–Burk equation [40]:

$$1/I_{ss} = 1/I_{max} + K_m^{app}/I_{max}C,$$

where I_{ss} is the steady-state current after the addition of substrate, I_{max} is the maximum current measured under saturated substrate conditions, and C is the bulk concentration of the substrate. According to the above equation, the K_m^{app} value for the enzymatic activity of the Nafion/Mb/IL/GCE to H_2O_2 was determined to be 22.6 μM. This shows that the immobilized Mb retains its bioelectrocatalytic activity and possesses a high biological affinity toward H_2O_2. This value was smaller than the corresponding values reported with other protein–RTIL film modified electrodes (Table 1), implying that Mb in this composite film is of a higher affinity to H_2O_2.

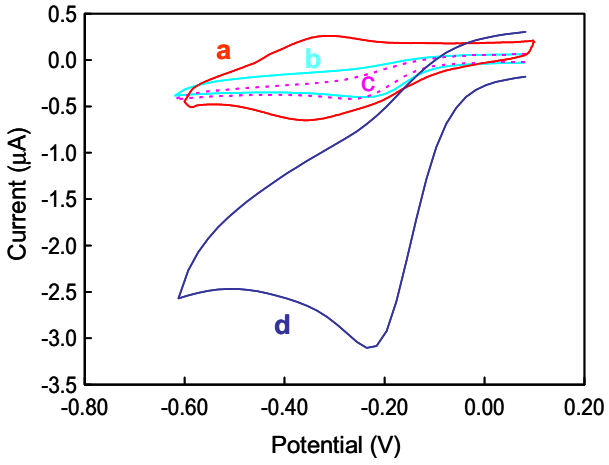

Fig. 5. CVs of electrodes in 0.1 M PBS (pH 7.0) at a scan rate of 0.1 V s^{-1}: (a and d) Nafion/Mb/IL/GCE; (b and c) Nafion/IL/GCE; (a and b) in the absence of H_2O_2; (c and d) in the presence of 0.1 mM H_2O_2.

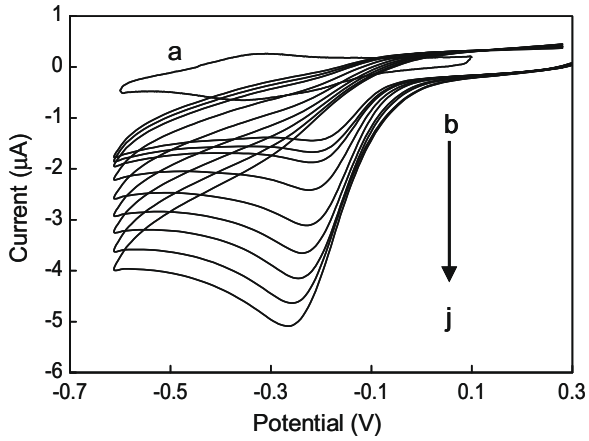

Fig. 6. Cyclic voltammetric response of the biosensor in PBS (pH 7.0) with different concentrations of H_2O_2 at a scan rate of 0.1 V s^{-1}. The H_2O_2 concentrations are as follows (a–j): 0, 10, 25, 50, 75, 100, 150, 200, 250, and 300 μM, respectively.

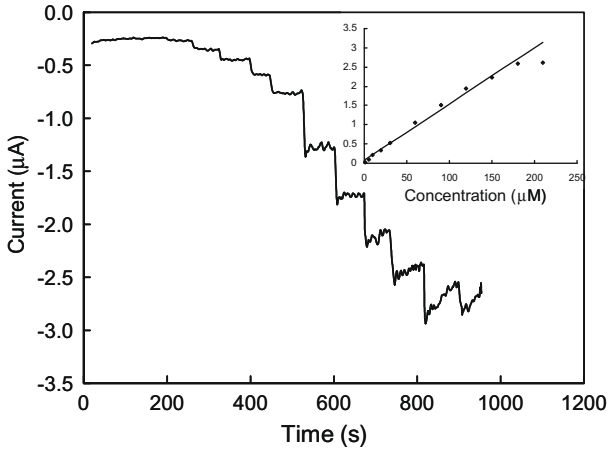

Fig. 7. Amperometric responses obtained with the biosensor on the successive addition of H_2O_2. The inset shows the relationship between catalytic currents and H_2O_2 concentrations. Conditions: 0.1 M PBS (pH 7.0); applied potential: −0.45 V (vs. Ag/AgCl).

Analysis of real samples

To evaluate the ability of the sensor for routine analysis, the sensor was applied to the determination of H_2O_2 in blood serum samples and also a commercial oxidant solution. The actual concentration of H_2O_2 in the oxidant solution was determined by the $KMnO_4$ titration method [41] and also by using the recommended procedure in this work. The results are presented in Table 2 (n = 3), which shows that there is a very good agreement between the results obtained for the commercial oxidant solution by the proposed sensor and those obtained by the $KMnO_4$ titration method. The results also show good reproducibility.

Table 1
Comparison of different protein–RTIL film modified electrodes for H_2O_2 determination

Electrode	Linear range (μM)	Detection limit (μM)	K_m^{app} (μM)	References
Mb/CILE	6.0–160	2	140	[40]
Nafion/MWCNTs/CILE	8.0–196	6	0.181	[42]
Mb/DNA/CILE	1.0–160	0.2	420	[43]
Nafion/nano-$CaCO_3$/Hb/CILE	8.0–440	–	120	[44]
Hb/IL/CILE	100–5000	4	7500	[45]
GCE/clay–IL/Mb	3.9–259	0.73	17.3	[25]
GNRs@SiO_2–Mb/RTIL–sol-gel/GCE	0.2–180	0.12	420	[23]
Mb–[BMIM][BF_4]–HA/GCE	2.0–270	0.6	290	[46]
HRP–chi-[BMIM][BF_4]/GCE	0.75–135	0.25	–	[24]
Cyt *c*/GNPs/RTIL/CNTs/GCE	50–1150	3	–	[47]
Nafion/Mb/IL/GCE	1.0–180	0.14	22.6	This work

Note. CILE, carbon ionic liquid electrode; MWCNTs, multiwalled carbon nanotubes; Hb, hemoglobin; GNRs, gold nanorods; [BMIM][BF_4], 1-butyl-3-methyl-imidazolium tetrafluoroborate; Cyt *c*, cytochrome *c*; GNPs, gold nanoparticles; CNTs, carbon nanotubes;HA, hyaluronic acid.

Table 2
Results of analysis of H_2O_2 in real samples.

Sample	Added (μM)	Found (μM)	Recovery	RSD (%)
Blood serum	–	0	–	–
	30	31.1	103.7	4.2
	60	60.3	100.5	3.8
	90	88.0	97.8	3.9
Oxidant[a]	–	85.2	–	3.6
	30	114.0	96.0	3.8
	60	145.0	99.7	4.1
	90	173.0	97.6	3.5

[a] The concentration found by the $KMNO_4$ titration method was 86.3 μM.

Repeatability, reproducibility, and stability of Nafion/Mb/IL/GCE

Nafion/Mb/IL/GCE was tested for stability while stored in an argon atmosphere at 4 °C. For this study, the CVs of Nafion/Mb/IL/GCE modified electrode were measured during several weeks periodically. The voltammetric data were quite stable within a month.

The repeatability of the catalytic response for six successive reductions of 100 μM H_2O_2 at Nafion/Mb/IL/GCE was estimated (PBS, pH 7.0) as 3.2%. The relative standard deviation (RSD) of the responses of five electrodes, made independently with the same method, was 5.6% at an H_2O_2 concentration of 100 μM. These indicate an efficient and reproducible immobilization process of Mb in Nafion/IL film. The selectivity of the sensor was also tested by studying the effects of foreign species on the determination of 100 μM H_2O_2. Here 1 mM of potential interfering substances, including glucose, sucrose, ethanol, citric acid, and ascorbic acid, were tested and no interference was observed.

Conclusions

In this article, an H_2O_2 amperometric sensor has been described by entrapping Mb in a composite film of IL and Nafion on GCE. The modified electrode could offer an elegant research platform for the direct electron transfer reaction of Mb. It was observed that the direct electron transfer rate between Mb and the electrode was greatly enhanced and that Mb retained its biological activity at the modified electrode. The biosensor has great capability in catalyzing the reduction of H_2O_2 and can be used as an amperometric sensor for the determination of H_2O_2. The developed biosensor exhibits a fast response to H_2O_2, has a lower detection limit than other IL–heme protein-based biosensors for the determination of H_2O_2, and is free from common interferences in H_2O_2 determination such as ascorbic acid. In addition, the biosensor also shows very good reproducibility and stability. In comparison with other methods used to fabricate biosensors by heme proteins, the electrode has excellent electrocatalytic function and its fabrication method is quite simple and convenient.

Acknowledgments

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